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Deletion of stem-loop 3 is compensated by second-site mutations within the Gag protein of human immunodeficiency virus type 1

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Abstract

Encapsidation of human immunodeficiency virus type 1 (HIV-1) RNA involves specific interactions between viral Gag proteins and viral RNA elements located at the 5′ untranslated region (UTR). These RNA elements are termed packaging (ψ) or encapsidation (E) signals and mainly comprise the stem-loop 1 (SL1) and SL3 RNA structures. We have previously shown that deletion of the SL1 sequences is compensated by second-site mutations within Gag. Similar studies are now extended to SL3 and the results demonstrate that deletion of this RNA structure is rescued by two point mutations, i.e., A11V in p2 and I12V in nucleocapsid (NC). These two compensatory mutations are different from those associated with the rescue of SL1 deletion, suggesting that SL1 and SL3 may bind to different residues of Gag during viral RNA packaging. Analysis of virion-derived RNA in native agarose gels shows that deletion of SL3 leads to decreases in both viral RNA packaging and dimerization. These defects are corrected by the compensatory mutations A11V and I12V. Yet, defects in viral RNA dimerization at an early stage that were caused by the SL3 deletion in the context of a viral protease-negative mutation cannot be overcome by these two suppressor mutations. Therefore, the positive effects of A11V and I12V on dimerization of the SL3-deleted RNA must have taken place at the maturation stage.

Introduction

Human immunodeficiency virus type 1 (HIV-1) selectively packages full-length viral RNA as its genome over a high background of cellular RNA in the cytoplasm. This process involves specific binding of HIV-1 Gag proteins to *cis*-acting viral RNA elements, termed ψ or encapsidation (E) signals. Early mutagenesis studies have mapped the E signals to a region located between the 5′ major splice donor (SD) site and the 5′ portion of the *gag* gene; this region was subsequently shown to contain two RNA stem-loop (SL) structures, termed SL3 and SL4 (Aldovini and Young, 1990; Clavel and Orenstein, 1990; Clever et al., 1995; Hayashi et al., 1992; Lever et al., 1989; Luban and Goff, 1994). Further studies revealed that RNA sequences located upstream of the 5′ SD site also affected RNA packaging (Kim et al., 1994); these include the TAR structure, the poly(A) hairpin, the U5-PBS RNA complex, and SL1 (Clever et al., 2002; Das et al., 1997, 1998; Harrich et al., 2000; Liang et al., 2000; McBride and Panganiban, 1997; McBride et al., 1996, 1997).

The nucleocapsid (NC) sequences of Gag protein function in trans to recognize the E signals and thereby direct RNA packaging (Aldovini and Young, 1990; Berkowitz and Goff et al., 1994, 1995; Dorfman et al., 1993; Poon et al., 1996). Of the multiple RNA structures within the 5′ noncoding RNA leader region, SL1, SL3, and SL4 bind to NC with high affinity, as shown by in vitro binding assays (Clever et al., 1995; Berkowitz and Goff, 1994; Berkowitz et al., 1993, Sakaguchi et al., 1993). In contrast, the TAR, U5-PBS, and poly(A) elements display low affinity for NC

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and thus may indirectly participate in RNA packaging by regulating viral RNA metabolism or by helping to correctly present the SL1, SL3, and SL4 structures to NC (Clever et al., 1995).

Aside from its role in viral RNA packaging, SL1 has also been defined as the dimerization initiation site (DIS). This function of SL1 is attributable to the palindrome feature of its loop sequence, which can trigger dimer formation through "Watson-Crick" basepairing (Clever et al., 1996; Laughrea and Jette, 1994, 1996; Marquet et al., 1994; Mujeeb et al., 1998; Muriaux et al., 1995, 1996b; Paillart et al., 1994, 1996; Skripkin et al., 1994). The importance of SL1 in RNA dimerization is also supported by experiments using mutated viral RNA derived from virus particles (Berkhout and van Wamel, 1996; Clever and Parslow, 1997; Haddrick et al., 1996; Laughrea et al., 1997; Shen et al., 2000). We have previously deleted the SL1 sequences in the context of full-length viral RNA genome and observed severe defects in both viral RNA packaging and dimerization (Shen et al., 2000). Long-term culture of the mutated viruses in permissive cells led to isolation of compensatory mutations within Gag protein that restored viral infectivity to near wild-type levels (Liang et al., 1998). Further analysis indicated that these compensatory mutations corrected the deficient viral RNA packaging but not RNA dimerization (Shen et al., 2000). It is thus proposed that viral RNA can be packaged at high efficiency in the monomeric form.

SL3 represents another major RNA packaging signal (Berkowitz et al., 1996). We have recently shown that disruption of the SL3 structure affected both RNA packaging and dimerization (Russell et al., 2003). To further understand the mechanisms underlying the regulatory roles of SL1 and SL3 in HIV-1 RNA packaging and dimerization, we have conducted a compensation study to determine the second-site mutations that can rescue the deleted SL3 sequences. The results show that the suppressor mutations for the SL3 deletion are different from those for the SL1 deletion, indicating that SL1 and SL3 may bind to Gag proteins in different ways during encapsidation of viral RNA.

Results

Long-term culture of the Δ(306–325) mutated viruses in MT-2 cells leads to the appearance of suppressor mutations in the gag coding region

HIV-1 RNA sequences at nucleotide (nt) 306 to 325 were deleted to remove the SL3 structure; the construct thus generated was termed Δ(306–325) (Fig. 1A). On the basis of the structure prediction by the M-fold program (Mathews et al., 1999; Zuker et al., 1999), this deletion does not disrupt the flanking RNA secondary structures including SL1 and SL2 (Fig. 1B). Following transfection of COS-7 cells, the mutated DNA construct Δ(306–325) generated wild-type levels of full-length as well as spliced viral RNA

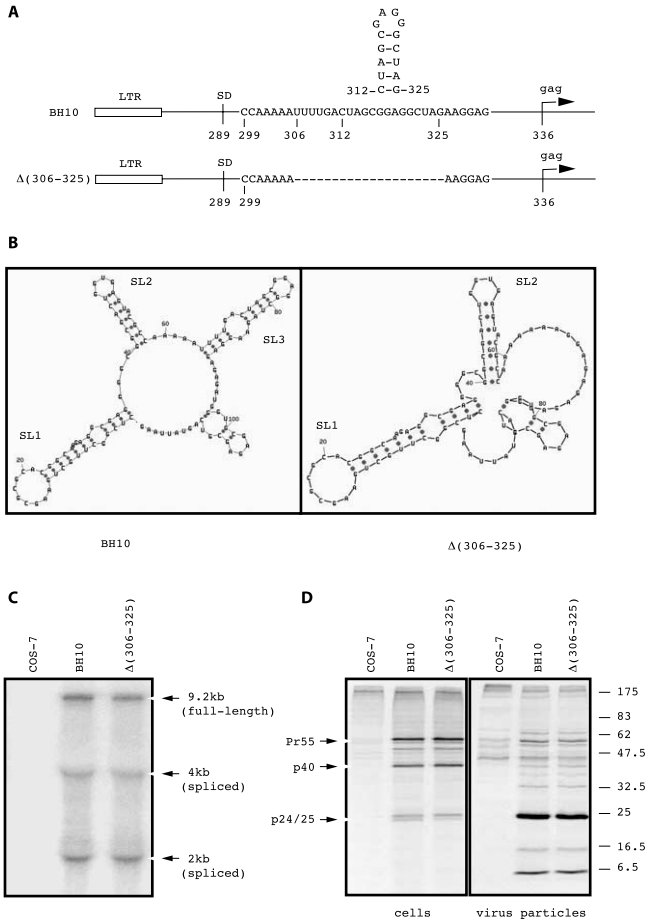

Fig. 1. (A) Schematic illustration of the Δ(306–325) deletion. Deleted sequences are indicated by dash lines. RNA secondary structures are shown for SL3. LTR, long terminal repeat; SD, splice donor. Numbering of nucleotides refers to the first nucleotide (nt) in the R region. (B) Effects of the Δ(306–325) deletion on viral RNA secondary structures. Both the wild-type BH10 and the Δ(306–325) mutated HIV-1 RNA sequences, spanning nucleotide positions 243 to 361, were subjected to secondary structure analysis through use of the M-fold program (Mathews et al., 1999; Zuker et al., 1999). Locations of the SL1, SL2, and SL3 structures are indicated. (C) Expression of viral RNA within transfected COS-7 cells. Total RNA was prepared from COS-7 cells that had been transfected with either the BH10 or Δ(306–325) DNA constructs, followed by separation on agarose gels. Northern blotting was performed to detect viral RNA through use of HIV-1 specific probes. The full-length (9.2 kb) as well as spliced (4 and 2 kb) forms of viral RNA are indicated on the right side of the gels. (D) Expression of viral proteins and production of virus particles. Transfected COS-7 cells were subjected to metabolic labeling through use of ^{35}S-methionine and ^{35}S-cysteine. The intracellular Gag protein and its derivatives were detected by immunoprecipitation using anti-HIV-1 p24 antibodies. ^{35}S-labeled virus particles in the culture fluids were directly assessed on Sodium dodecyl sulfate–12% polyacrylamide gels and visualized by exposure to x-ray films. Positions of the protein markers (kDa) are indicated on the right side of the gels.

as shown by the results of Northern blots (Fig. 1C). This indicates that the SL2 RNA structure, which contains the splice donor signal, is not affected by the Δ(306–325) deletion and thus allows normal splicing of viral RNA. In support of these results of viral RNA analysis, wild-type levels of both intracellular Gag proteins and extracellular

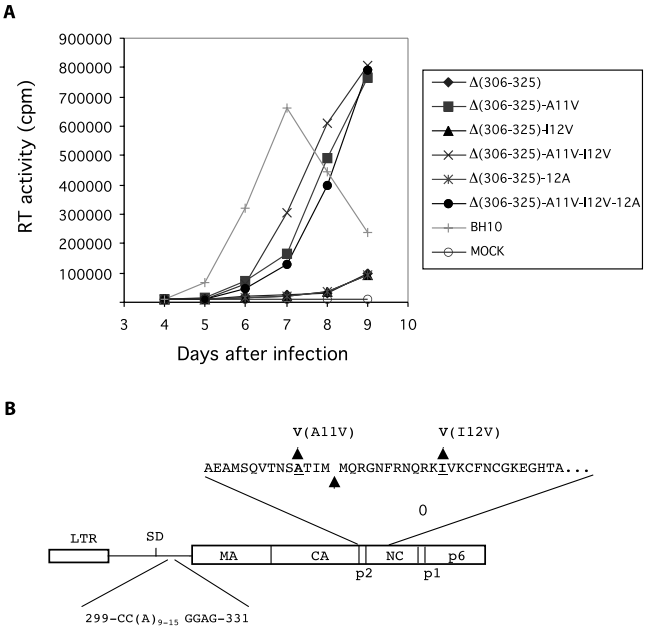

Fig. 2. Compensation of the crippled replication of the Δ(306–325) mutated viruses by second-site mutations within the Gag protein. (A) Replication of mutated and wild-type viruses in MT-2 cells. Virus growth was monitored by measuring reverse transcriptase (RT) activity of culture fluids at various times. Mock infection represents exposure of MT-2 cells to heat-inactivated wild-type viruses. (B) Second-site mutations identified in the Δ(306–325) mutated viruses after long-term culture in MT-2 cells. The changed amino acid residues in the p2 and NC domains are underlined. The arrow indicates the protease cleavage site between p2 and NC.

virus particles were detected after transfection of this mutated DNA construct into COS-7 cells (Fig. 1D).

The infectivity of the virus particles that were generated either by the wild-type BH10 or the mutated Δ(306–325) DNA constructs was then studied through infection of MT-2 cells. The results show that growth of the Δ(306–325) mutant viruses was significantly delayed in comparison to the wild-type virus (Fig. 2A). The mutated viruses were further cultured in MT-2 cells over multiple passages until wild-type replication phenotypes were observed. Sequencing of proviral DNA at this stage indicated the retention of the original SL3 deletion and the emergence of novel second-site mutations in the *gag* coding region, i.e., an A11V amino acid substitution in p2 and an I12V amino acid substitution in NC (Fig. 2B). In addition, insertions of a number of As were seen between nt positions 305 and 306 in the noncoding RNA leader region. Site-directed mutagenesis experiments were then performed to insert these second-site mutations into the Δ(306–325) construct to determine whether these new mutations were sufficient and necessary to restore the infectivity of Δ(306–325) to wild-type levels. The results of infection assays showed that the A11V mutation in p2 markedly increased the infectiousness of Δ(306–325); the I12V mutation alone barely augmented viral replication, but could synergistically increase viral growth together with A11V (Fig. 2A). The addition of A residues did not increase the infectivity of Δ(306–325), either alone or together with A11V and I12V (data are shown only in regard to the addition of 12 As) (Fig. 2A). Therefore, second-site mutations in both p2 and NC apparently played key roles in rescue of the defective replication of Δ(306–325).

The A11V mutation in p2 and the I12V mutation in NC increase levels of RNA packaging and dimerization in the Δ(306–325) mutated viruses

SL3 is known to direct specific packaging of HIV-1 genomic RNA. Thus, we hypothesize that the A11V and I12V mutations must have overcome deficits in packaging caused by the Δ(306–325) deletion. To test this hypothesis, viral RNA was prepared from virus particles through protease K digestion and phenol:chlorophorm extraction in the presence of 100 mM NaCl, fractionated on native agarose gels, and analyzed by Northern blotting. The results showed that the Δ(306–325) deletion led to severe reductions not only in the levels of total viral RNA but also in the relative levels of dimeric RNA (Fig. 3, lane 1). Following combination with the Δ(306–325) deletion, A11V gave rise to a measurable increase of viral RNA levels (Fig. 3, lane 2), while the I12V mutation alone was not effective in this regard (Fig. 3, lane 3). However, A11V and I12V together restored viral RNA packaging in Δ(306–325) to wild-type levels and also significantly increased dimer contents in the mutant virus particles (Fig. 3, lane 4). Therefore, A11V and I12V acted synergistically to rescue the deficient RNA packaging and dimerization in Δ(306–325).

We also examined the effects of additional inserted As on viral RNA dimerization and packaging in Δ(306–325). The results of Fig. 3 show that neither 9 nor 12 As, inserted at nt 305, led to any increase in the total levels of Δ(306–325) RNA (lanes 5 and 6). However, insertion of 15 As restored wild-type RNA packaging; yet, the majority of viral RNA exhibited a monomeric form (lane 7). Again, the A11V and I12V mutations were jointly able to overcome most of the deficits in RNA packaging and dimerization caused by the Δ(306–325) deletion in the presence of the inserted 15 As (lane 8).

We next asked whether the corrected RNA dimers in the Δ(306–325)-A11V-I12V recombinant viruses possessed wild-type thermostability. To pursue this subject, viral RNA samples were treated at various temperatures in the presence of 100 mM NaCl before separation on native agarose gels. We first examined dimers retained in the Δ(306–325) mutated viruses. The results of Fig. 4 show that these RNA dimers dissociated over a range of temperatures similar to those seen with wild-type dimers. When the Δ(306–325)-A11V-I12V RNA dimers were analyzed, the same dissociation temperatures were again observed (Fig. 4). Therefore, the Δ(306–325) dimers that were formed in the presence of A11V and I12V mutations exhibit wild-type thermostability.

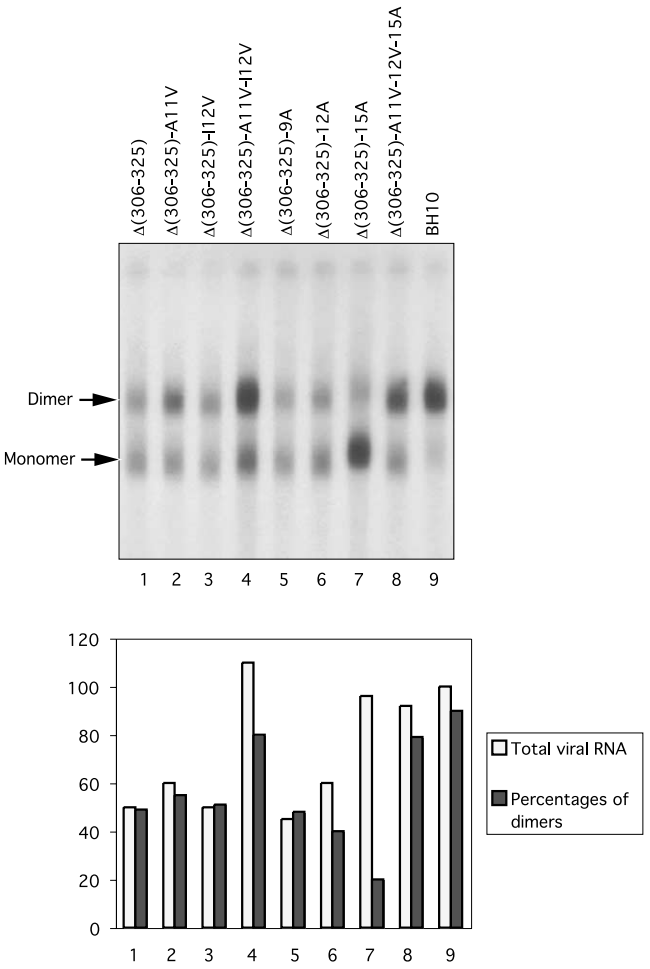

Fig. 3. Effects of the A11V and I12V second-site mutations on deficient RNA packaging and dimerization of Δ(306–325). Viral RNA was prepared from virus particles by phenol-chloroform extraction. An amount of viral RNA equivalent to 150 ng of p24 (CA) antigen was loaded onto native agarose gels for each construct. Following electrophoresis, viral RNA was transferred onto nylon membranes and further detected by human immunodeficiency virus type 1 (HIV-1) DNA probes. Positions of dimers and monomers are indicated on the left side of the gels. Intensities of RNA signals were calculated by using the NIH Image program and the results are summarized in the graph. The total levels of wild-type RNA in BH10 were arbitrarily set at 100. The relative proportion of dimers for each construct was also determined and the results are shown in the graph. The data shown are from one representative experiment.

The A11V and I12V substitutions do not correct defective RNA dimerization caused by the Δ(306–325) deletion in the context of protease-negative viruses

HIV-1 RNA is initially packaged as immature dimers that are further converted into mature forms upon release of the NC protein by protease (PR) cleavage of the Gag protein (Fu et al., 1994). The immature dimers are readily detected within the protease-negative virions and exhibit a slightly slower mobility during electrophoresis as well as lower stability in comparison to the mature dimers that are associated with the wild-type mature virus particles. We wished to determine whether the Δ(306–325) deletion had exerted adverse impacts on dimerization at the immature dimer stage. To pursue this subject, the Δ(306–325) deletion was combined with a point mutation D25A that eliminates the enzymatic activity of the HIV-1 PR (Morin et al., 1998), and the construct thus generated was termed Δ(306–325)-PR$^-$. The D25A substitution was also inserted into wild-type BH10 to generate BH-PR$^-$. In the case of BH-PR$^-$, a significant amount of viral RNA was observed as immature dimers that migrated at a slower rate than did the wild-type RNA dimers in BH10 (Fig. 5). This indicates that the immature dimers are in a relatively loose conformation compared to the mature dimers (Fu et al., 1994). In contrast, RNA derived from Δ(306–325)-PR$^-$ virus was present almost exclusively in monomeric form (Fig. 5). These findings suggest that the Δ(306–325) RNA is defective in the formation of immature dimers during virus assembly.

Next, we asked whether the compensatory mutations A11V and I12V were able to correct this early dimerization defect. Accordingly, Δ(306–325), A11V, and I12V were combined with the PR mutation D25A to yield a construct Δ(306–325)-R-PR$^-$. The results showed that levels of RNA immature dimers in this latter virus were drastically lower than those associated with BH-PR$^-$ (Fig. 5). These data, together with those shown in Fig. 3, lead to the conclusion that rescue of deficient dimerization by A11V and I12V must have taken place during viral maturation following PR cleavage.

Discussion

In the present study we have identified two second-site mutations within the Gag protein that can rescue the deleted

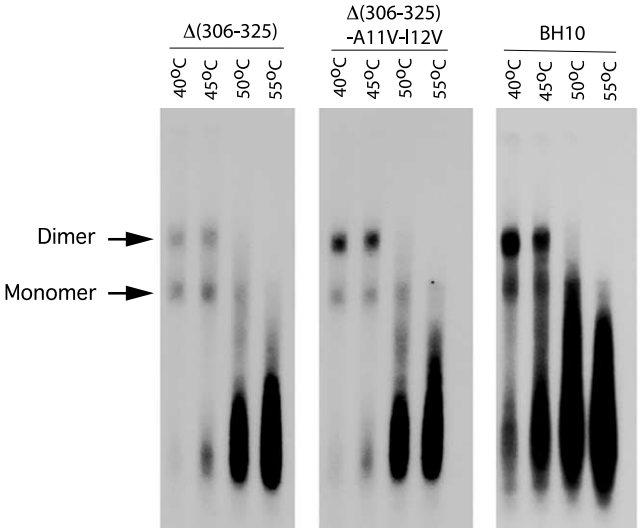

Fig. 4. Thermostability of RNA dimers prepared from the Δ(306–325), Δ(306–325)- A11V-112V, and BH10 viruses. Viral RNA samples were treated at different temperatures (i.e., 40°C, 45°C, 50°C, and 55°C) in a buffer containing 100 mM NaCl prior to separation on native agarose gels.

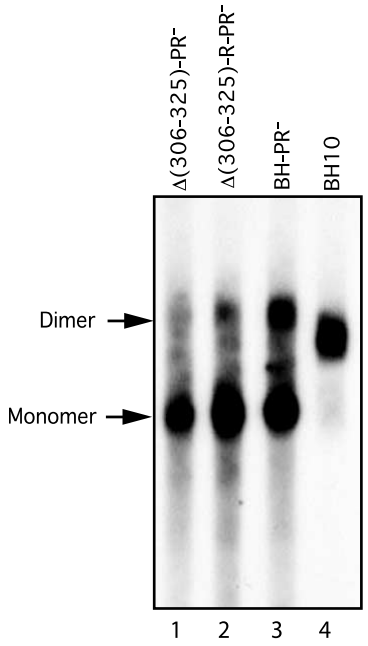

Fig. 5. Effects of the Δ(306–325) deletion on RNA dimerization in the PR$^-$ virus. Construct Δ(306–325)-R-PR$^-$ contains the Δ(306–325) deletion, the second-site mutations A11V and I12V, as well as PR mutation D25A.

SL3 sequences. Interestingly, the two mutations, i.e., A11V in p2 and I12V in NC, are distinct from those previously characterized for the deleted SL1 sequences, which include substitutions T12I in p2 and T24I in NC (Liang et al., 1998). This difference suggests that even though both SL1 and SL3 participate in RNA packaging via binding to Gag (Clever et al., 1995), the detailed RNA-protein interactions may vary. In support of this notion, the A11V and I12V mutations could not rescue the SL1-deleted viruses in regard to its crippled viability as well as defective RNA packaging and dimerization (data not shown).

Compensation of the deleted SL1 and SL3 sequences by their relevant suppressor mutations involves restoration of wild-type RNA packaging. Yet, in contrast to mutations A11V and I12V that also corrected the defective dimerization caused by the deleted SL3 sequences, mutations T12I and T24I could not fix the defective dimers associated with the deleted SL1 sequences (Shen et al., 2000). Thus, SL1 and SL3 may regulate viral RNA dimerization via distinct mechanisms. Results of cell-free assays show that SL1 is able to form dimers spontaneously without the assistance of any viral or cellular proteins by virtue of its palindrome loop sequence (Clever et al., 1996; Laughrea and Jette, 1994, 1996; Marquet et al., 1994; Mujeeb et al., 1998; Muriaux et al., 1995, 1996b; Paillart et al., 1994, 1996; Skripkin et al., 1994); in contrast, short synthetic viral RNA fragments containing the SL3 sequence do not dimerize (Sundquist and Heaphy, 1993). Antisense DNA oligonucleotides, targeting either SL1 or SL3, were able to block dimerization of synthetic viral RNA fragments. However, the SL3-antisense molecules bound to RNA dimers while the SL1-antisense molecules did not, perhaps because SL1 already forms "Watson-Crick" basepairs within dimers and SL3 may have been involved in other types of molecular interactions (Zhang et al., 1997).

Rescue of the deleted SL3 sequences by second-site mutations in p2 (A11V) and NC (I12V) involves increases in the levels of both RNA packaging and dimerization. It is thus possible that increased dimerization is a result of optimized RNA packaging. Although we cannot rule out this possibility at this stage, studies performed with PR-negative viruses indicate that the A11V and I12V mutations cannot rescue defective immature dimers caused by the Δ(306–325) deletion (Fig. 5). Together with the fact that these two substitutions can increase packaging of Δ(306–325) RNA to wild-type levels (Fig. 3), we conclude that to the least extent, the early defects in dimerization caused by Δ(306–325) prior to PR cleavage are irrelevant to the deficient packaging of Δ(306–325) RNA.

Since SL1 and SL3 are closely located within viral RNA genome, it is possible that deletion of SL3 may have interfered with the folding of the SL1 structure, and thereby indirectly affected dimerization. This seems unlikely, since the results of RNA secondary structure prediction by the M-Fold program show that deletion of SL3 still allows the correct folding of SL1 as well as the other structures within the noncoding RNA leader region (Fig. 1B). Therefore, although results of computer modeling need to be further verified by structural probing experiments, it is highly possible that the Δ(306–325) deletion may have disturbed RNA dimerization through mechanisms other than disruption of the SL1 structure.

We hypothesize that the involvement of SL3 in RNA dimerization may be related to its NC-binding activity (Berkowitz et al., 1993, 1994; Clever et al., 1995; Sakaguchi et al., 1993). NC has been shown to promote dimerization of synthetic viral RNA fragments (Feng et al., 1996; Muriaux et al., 1996a), and mutations within NC zinc finger motifs led to deficient viral RNA dimerization within virions (Laughrea et al., 2001). Therefore, the role of SL3 in dimerization may partially be due to its function in the specific recruitment of NC onto viral RNA.

Notably, compensation of mutated RNA packaging signals (e.g., SL1 or SL3) involves second-site mutations in the p2 sequence (e.g., T12I and A11V); this indicates a potential role of p2 in recognition of viral RNA during viral assembly. Indeed, presence of HIV-1 p2 sequence, together with NC, in an HIV-1/HIV-2 chimeric virus significantly augments specific packaging of HIV-1 RNA; this constitutes the first evidence that p2 may play important roles in HIV-1 RNA packaging (Kaye and Lever, 1998).

Together with our previous findings obtained with the deleted SL1 sequences (Liang et al., 1998), this study further demonstrates that HIV-1 is capable of surviving partially removed RNA packaging signals by modifying the Gag sequences. Presumably, these modified Gag proteins

must have possessed wild-type levels of binding affinity for the mutated viral RNA and thus restored wild-type RNA packaging.

Materials and methods

Plasmid construction

The BH10 infectious HIV-1 cDNA clone was employed as starting material. Mutagenesis studies were performed on the basis of polymerase chain reactions (PCR) using *Pfu* enzyme with 3′ to 5′ proof-reading activity (Stratagene, La Jolla, CA). The primers used were purchased from Invitrogen (Burlington, Ontario, Canada). The Δ(306–325) deletion was engineered through use of primer pairs pΔSL3(5′-CTGAAGCGCGCACGGCAAGAGGCGAGGGGCGGCGACTGGTGAGTACGCCAAAAAAAGGAGAGAGATGGG-3′, [nt 252 to 340])/pAPA-A(5′-CCTAGGGGCCCTGCAATTTCTG-3′ [nt 1562 to 1541]). The PCR products were digested with restriction enzymes *BssH*II and *Apa*I (Invitrogen) and used to replace the same regions in BH10.

The A11V amino acid substitution in p2 was created by using a PCR-Script Amp cloning kit (Stratagene) through use of primers p2-S2 (5′-GTAACAAATTCAGTTACCATAATGATGCAG-3′ [nt 1443 to 1472])/p2-A2 (5′-GCATCATTATGGTAACTGAATTTGTTACTTGG-3′ [nt 1470 to 1439]). The I12V mutation in the NC sequence was engineered by PCR by using primer pair pE (5′-GGAACCAAAGAAAGGTTGTTAAGTGTTTC-3′ [nt 1486 to 1514])/pNC-A (5′-TTAGCCTGTCTCTCAGTACAATC-3′ [nt 1630 to 1608]). The PCR product was used as a primer in a second round of PCR together with primer pSph-S (5′-AGTGCATCCAGTGCATGCAGGGCC-3′ [nt 977 to 1000]). The final PCR product was digested with *Sph*I and *Apa*I and inserted into Δ(306–325). The positions of primers are in relation to the first nt of the 5′ R region. The mutations generated were confirmed by sequencing.

Cell culture, transfection, and infection

COS-7 and MT-2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) and RPMI 1640 medium, respectively, supplemented with 10% fetal calf serum (Invitrogen). Transfection of COS-7 cells was performed with Lipofectamine (Invitrogen). Progeny virus in culture fluids were collected 48 h after transfection and quantified by measuring levels of HIV-1 CA (p24) antigen (Ag) by enzyme-linked immunosorption assays (ELISA) (Vironostika HIV-1 Antigen Microelisa System, Organon Teknika Corporation, Durham, NC).

Viruses containing 3 ng of p24 were used to infect 5×10^5 MT-2 cells in 2 ml of RPMI 1640 medium. After 2 h, cells were washed twice to remove unbound viruses and were grown in 10 ml of complete medium. Growth of viruses was monitored by measuring reverse transcriptase (RT) activity of culture fluids at various times.

Analysis of viral proteins

COS-7 cells were transfected for 20 h, followed by starvation in DMEM without Met and Cys at 37°C for 2 h. Total cellular proteins were then radiolabeled by ^{35}S-Met and ^{35}S-Cys (ICN, Irvine, CA) at a concentration of 0.1 mCi/ml for 30 min at 37°C. The labeled cells were either lysed immediately after labeling or cultured for an additional hour in complete DMEM before being lysed in NP-40 lysis buffer. Cell lysates were incubated with monoclonal antibodies (mAbs) against HIV-1 p24 and the Ag:mAb complexes were absorbed with protein A-linked Sepharose 4B (Amersham Pharmacia Biotech., Baie d'Urfe, Quebec, Canada). After extensive washing, the immunoprecipitated viral proteins were fractionated on sodium dodecyl sulfate (SDS)-12% polyacrylamide gels and visualized by exposure to x-ray films (Kodak, Rochester, NY). The ^{35}S-labeled virus particles in the culture fluids were collected through centrifugation and directly analyzed by running on SDS-12% polyacrylamide gels.

Analysis of viral RNA by Northern blots

Progeny viruses generated by transfected COS-7 cells were first clarified by centrifugation in a Beckman GR-6S centrifuge at 3000 rpm for 30 min at 4°C and then pelleted through a 20% sucrose cushion by ultracentrifugation in a Beckman XL-80 ultracentrifuge using an SW41 rotor at 40,000 rpm for 1 h at 4°C. Virus pellets were suspended in 300 μl of TN buffer, a 2-μl portion was removed for p24 determination, and the remaining viruses were digested with 100 μg/ml protease K in the presence of 1% SDS, 10 mM EDTA, 100 mM NaCl, and 50 μg of yeast tRNA for 20 min at 37°C. Samples were then extracted twice with phenol: chlorophorm:isoamlyalcohol (25:24:1) and once with chloroform. Viral RNA was precipitated in 2.5 volumes of 95% ethanol. RNA pellets were washed with 70% ethanol and dissolved in TE buffer. An amount of viral RNA equivalent to 150 ng of HIV-1 p24 was electrophoresed on 0.9% native agarose gels in 1 × TBE buffer at 100 V for 4 h at 4°C. The RNA was then transferred to a nylon membrane and analyzed by Northern blots using ^{32}P-α-dCTP (ICN) labeled HIV-1 DNA probes. To determine the thermostability of viral RNA dimers, RNA samples were treated at various temperatures (e.g., 40°C, 45°C, 50°C, and 55°C) for 10 min in a buffer containing 100 mM NaCl before being separated on native agarose gels.

To assess the expression of both full-length and spliced forms of viral RNA, total RNA was extracted from transfected COS-7 cells and separated on 1% agarose gels containing formaldehyde as a denaturant. RNA molecules were then transferred onto a nylon membrane and hybridized to HIV-1 probes as described above.

Identification of second-site mutations

MT-2 cells that had been infected with mutated viruses were cultured for prolonged periods until formation of syncytia and high levels of RT activity were observed. The infectious virus particles were further used to infect fresh MT-2 cells until wild-type replication kinetics were seen. Cellular DNA was then extracted from infected MT-2 cells and subjected to PCR and sequencing to identify newly emerged second-site mutations in proviral DNA (Liang et al., 1998).

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